



10/501682

Rec'd PCT/PTO 16 JUL 2004



INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport

South Wales
NP10 8QQ

07 MAR 2003

WIPO

PCT

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed

Dated

5 February 2003

BEST AVAILABLE COPY

Patents Form 1/77

Act 1977
(page 16)17 JAN 02 1688397-1/003312
P01/7700 0.00-0200929.8

THE PATENT OFFICE
A
LONDON

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

16 JAN 2002

16 JAN 2002

The Patent Office

Cardiff Road
Newport
South Wales
NP10 8QQ

1. Your reference

PC/GOW/DH/P11902GB

2. Patent application number

(The Patent Office will fill in this part)

0200929.83. Full name, address and postcode of the or of each applicant (underline all surnames)

The University Court of The University of
Glasgow
University Avenue
2 The Square
Glasgow
G12 8QQ

773853002

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

UK

4. Title of the invention

Tissue Rejection

5. Name of your agent (if you have one)

CRUIKSHANK & FAIRWEATHER
19 ROYAL EXCHANGE SQUARE
GLASGOW
G1 3AE

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

Patents ADP number (if you know it)

547002 ✓

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number

Country

Priority application number
(if you know it)

Date of filing
(day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

Date of filing
(day / month / year)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if)

YES

- a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
(See note (d))

Patents Form 1/77

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form

Description

29 /

Claim(s)

Abstract

Drawing(s)

3 only

10. If you are also filing any of the following, state how many against each item.

Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (Patents Form 7/77)

Request for preliminary examination and search (Patents Form 9/77)

Request for substantive examination (Patents Form 10/77)

Any other documents (please specify)

11.

I/We request the grant of a patent on the basis of this application.

Signature

Cruikshank & Fairweather

Date

16/1/02

12. Name and daytime telephone number of person to contact in the United Kingdom

Dr. Paul Chapman (0141) 221 5767

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

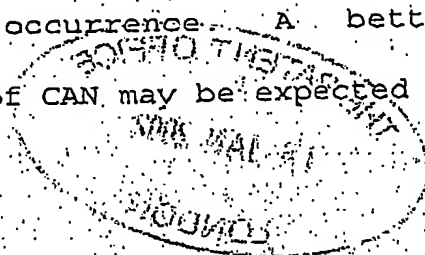
Notes

- a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 08459 500505.
- b) Write your answers in capital letters using black ink or you may type them.
- c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.
- d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.
- e) Once you have filled in the form you must remember to sign and date it.
- f) For details of the fee and ways to pay please contact the Patent Office.

TISSUE REJECTION

The present invention relates to a preparation for use in the evaluation of the risk of tissue rejection of donor tissues. The invention further relates to preparations for use in suppressing tissue rejection, and still further to methods of assessing and suppressing tissue rejection of donor tissues.

Transplantation is now the preferred treatment for cases of chronic renal failure. There is however a large disparity between the number of potential transplant recipients and the number of available donor kidneys. This disparity is also evident in all other transplant cases. Failure of transplanted or grafted tissues (the term 'tissues' as used herein will be understood to refer equally to cells and organs) is therefore of extreme clinical, economic, and emotional significance on recipients of donor tissue. In terms of kidney transplants, chronic allograft nephropathy (CAN) represents the most common cause of graft loss, once patient death has been accounted for. Unfortunately, the pathogenesis of CAN, at present, remains poorly understood and little progress has been made in reducing its occurrence. A better understanding of the pathogenesis of CAN may be expected to



yield new light on how to reduce the occurrence of CAN
(Halloran et al, 1999).

Previous hypotheses to explain the pathogenesis of CAN
have concentrated on possible immune-based explanations.

5 However, these now appear unlikely, since the majority of
~~risk factors for CAN are non-immune, and the widespread~~
introduction of new immunosuppressants in the 1980s did not
lead to a significant drop in the occurrence of CAN (Paul,
1999).

10 It is among the objects of embodiments of the present
invention to provide preparations for reducing the
occurrence of CAN in donor tissues. This is achieved, in
part, by the surprising findings of the present inventors
that a number of senescence-associated genes (SAGs) have
15 been found to show altered expression in tissues
susceptible to CAN.

It has been suggested that one possible cause of CAN
may be accelerated ageing, where the accumulated burden of
injury and age exhausts the ability of key cells to repair
20 and remodel, and therefore to retain tissue integrity
(Halloran et al, 1999). The present invention is based upon
a model for CAN in which damage to the tissue leads to
altered expression of genes involved in cellular

senescence, resulting in CAN. The particular genes which are implicated in cellular senescence are the telomere-binding proteins, as discussed below. It has been surprisingly found by the present inventors that specific telomere-binding proteins show increased expression in kidneys that have been subject to CAN when compared to normal tissues. Surprisingly also, the present inventors have also found decreased expression of a given SAG in tissues derived from a cadaveric donor, when compared to those derived from a living donor, or indeed those from living donors that have subsequently undergone CAN.

According to a first aspect of the present invention, there is provided a method of screening mammalian donor tissues for predisposition to rejection, the method comprising the steps of determining the level of expression of an endogenous telomere-binding protein in the donor tissue, and comparing the determined level to a reference level of expression, altered levels of expression in the donor tissue being indicative of a predisposition to rejection.

Of course, it will be appreciated that the present method alone does not enable a medical practitioner to definitively assess whether or not a given donor tissue is

suitable for transplant. Many other factors must be taken into consideration, for example, the age and general health of both the donor and the recipient, the urgency of the transplant, immunological factors, and so forth. The skilled person will be aware of the many relevant factors which will be considered. Nonetheless, the method of the

present invention is intended to be a useful addition to the skilled person's other considerations and techniques.

Preferably the method is carried out on a donor tissue previously removed from a donor body. Alternatively this method may be carried out on a donor tissue post-transplant in order to provide data for the determination and suitability of therapeutic intervention or change of therapeutic regime.

The mammalian tissue may be human or animal tissue, such as porcine tissue.

In a particularly preferred embodiment the tissue being tested is renal tissue.

The method may comprise determining the expression level of one or more telomere binding proteins; preferably at least two, and more preferably at least three. This allows assessment to be made on the basis of a number of diagnostic proteins, to reduce the risk of false positives.

5

Preferably the telomere binding protein is selected from the group comprising G22P1, XRCC5, hPOT1, and SIRT2, and their homologues or analogues. These genes and their proteins are described and characterised in the publications listed in the OMIM database at:

G22P1 - <http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispnim?152690>

XRCC5 - <http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispnim?194364>

10 hPOT1 - <http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispnim?606478>

SIRT2 - <http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/dispnim?604479>

Where the telomere binding protein is one or more of G22P1, XRCC5, and hPOT1, and homologues thereof, an increase in expression of these proteins is taken as indicative of a predisposition to rejection. Where the telomere binding protein is SIRT2 or a homologue thereof, a decrease in expression of this protein is indicative of a predisposition to rejection in tissue taken from a cadaveric source.

Alternatively, another telomere binding protein or senescence associated gene may be selected; for example,

the protein or gene may be selected from the group comprising Rif1, Rif2, Rap1, SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, Est1, Est2, TLG1, cdc13, A26, ATM, HDAC1, hSEI1, hTEP1, HuCds1, MYC, NEK2, p21, PIN2, TNKS, TERC, hTERT, TOP2A, TOP2B, TP53, TRF1, TRF2, WRN. Full details of such genes are given at the following location.

<http://www.genlink.wustl.edu/telldb/ptelldb/ptelldb3.html>.

The step of determining the level of expression of the telomere binding protein may be conducted by means of detection of the protein with antibodies thereto; the antibodies may themselves be labelled in some way (for example, radiolabelling, fluorescent labelling, or the like), or the antibodies may be subsequently detected using additional reagents, for example in the form of an ELISA assay well known to those of skill in the art.

Alternatively, the levels of expression of mRNA encoding the protein may be determined, using, for example, Northern blotting, polymerase chain reaction (PCR) detection, in situ hybridisation, microarray or macroarray technology, or the like.

The reference level of expression may be a predetermined reference level, or may be determined substantially at the same time as the donor tissue level of

7
expression; for example, from tissues of the recipient or
of a control subject. Preferably the reference level is
obtained from a healthy tissue sample.

According to a second aspect of the present invention,
5 there is provided a kit for screening mammalian donor
tissues for predisposition to rejection, the kit comprising
reagents for determining the level of expression of an
endogenous telomere binding protein in the donor tissue.

Preferably the kit comprises PCR primers for detection
10 of mRNA encoding the telomere binding protein. Preferably
the protein is selected from the group comprising G22P1,
XRCC5, hPOT1, and SIRT2, or homologues and analogues
thereof. Alternatively, another telomere binding protein or
senescence associated gene may be selected; for example,
15 the protein or gene may be selected from the group
comprising Rif1, Rif2, Rap1, SIRTs 1,3,4,5, Est1, Est2,
TLG1, cdc13, A26, ATM, HDAC1, hSEPI, hTEPI, HuCds1, MYC,
NEK2, p21, PIN2, TNKS, TERC, hTERT, TOP2A, TOP2B, TP53,
TRF1, TRF2, WRN.

20 Alternatively, the kit may comprise antibodies to the
telomere binding protein. The kit may further comprise
detection reagents for detection of bound antibodies;
alternatively or in addition the antibodies themselves may

be labelled to allow direct or indirect detection thereof.
For example, radioactive or fluorescent labels may be used.
A further alternative for the kit is that it may provide
probes (such as DNA, RNA, or chimerical combinations of
5 both or either) together with a further agent, for example
~~a proteinaceous agent such as an antibody or other agent~~

According to a further aspect of the present
invention, there is provided a method of treatment of a
mammalian donor tissue to reduce the risk of rejection, the
10 method comprising the step of treating the tissue with an
agent to enhance the activity, half-life or expression
level of an endogenous telomere binding protein.
Alternatively, or in addition, the tissue may be treated
with an agent to enhance the effective functionality of the
15 endogenous telomere binding protein.

It is believed that the increased expression levels of
certain telomere binding proteins reflects increased levels
of cell damage in rejection-susceptible tissues. Without
wishing to be bound by theory, it is believed that as
20 cellular DNA is damaged, existing telomere binding proteins
are recruited away from the telomere to the site of the
damage for repair purposes. Increased expression of these
proteins results as the cell attempts to maintain

sufficient protein at the telomeres; this increased expression is detectable as discussed above. However, if insufficient protein is present, telomere damage and cell death or degradation may occur. Thus, further increasing the expression or otherwise assisting the functionality of these proteins may be expected to act to repair the damage and so reduce the risk of tissue rejection.

The donor tissue may be human or animal.

Conveniently the tissue is renal tissue.

10 Preferably the protein is selected from the group comprising G22P1, XRCC5, hPOT1, and SIRT2, and homologues or analogues thereof. The protein may instead comprise an active site homologous to that of G22P1, XRCC5, hPOT1, or SIRT2. Alternatively, another telomere binding protein or
15 senescence associated gene may be selected; for example, the protein or gene may be selected from the group comprising Rif1, Rif2, Rap1, SIRTs 1,3,4,5, Est1, Est2, TLG1, cdc13, A26, ATM, HDAC1, hSEPI, hTEPI, HuCds1, MYC, NEK2, p21, PIN2, TNKS, TERC, hTERT, TOP2A, TOP2B, TP53,
20 TRF1, TRF2, WRN.

One or more protein activity, expression levels or half-life may be enhanced; preferably two or more; more preferably three or more proteins are enhanced.

10

The enhancement of gene expression levels may be achieved via a number of strategies including : gene therapy; promoter / enhancer knock in; promoter / enhancer induction; gene replacement; and the like. These methods
5 are not an exhaustive list of the full range of such techniques which typically are known to those of skill in the art.

According to a further aspect of the present invention, there is provided the use of a mammalian
10 telomere binding protein in the preparation of a medicament for suppression of rejection of a donor mammalian tissue.

The tissue is preferably renal tissue.

The protein is preferably selected from the group comprising G22P1, XRCC5, hPOT1, and SIRT2, and homologues
15 and analogues thereof. Alternatively, another telomere binding protein or senescence associated gene may be selected; for example, the protein or gene may be selected from the group comprising Rif1, Rif2, Rap1, SIRTs 1,3,4,5, Est1, Est2, TLG1, cdc13, A26, ATM, HDAC1, hSEP1, hTEP1,
20 HuCdsl, MYC, NEK2, p21, PIN2, TNKS, TERC, hTERT, TOP2A, TOP2B, TP53, TRF1, TRF2, WRN.

According to a still further aspect of the present invention, there is provided a non-human mammalian donor

11

tissue in which the expression of at least one endogenous telomere binding protein has been enhanced.

Such tissues may be useful in xenotransplantation, to lessen the risk of rejection of the donor tissue.

5 Enhancement may be as a result of gene therapy, or transgenesis or molecular manipulation of cells subsequently used for nuclear transplantation. Promoter or enhancer knock in or induction element knock in are also applicable for these purposes.

10 These and other aspects of the present invention will now be described with reference to the accompanying drawings, in which:

Figure 1 shows the relative levels of expression of G22P1, XRCC5, hPOT1, and SIRT2 mRNA from different types of
15 renal tissue; and

Figure 2 shows the results of a statistical analysis of the results of Figure 1.

Before discussing these Figures, for a better understanding of the invention, some further background
20 detail will be given on the senescence of cells and the action of the relevant proteins.

Telomere instability

Telomeres are specialised structures found at the end

of eukaryotic chromosomes consisting of simple repetitive DNA, which in mammals comprises the sequence (TTAGGG)_n.

Without specialised structures to cap telomeres and to replicate them during cell division, the telomeres gradually shorten over time. It is generally believed that shortening of telomere length through repeated cell divisions over time contributes to the ageing and senescence of somatic cells (reviewed in Shiels 1999).

The telomere capping function appears to be mediated by a combination of a unique tertiary structure and specific telomere binding proteins. Electron microscopy has shown that the mammalian telomere takes the form of a loop, termed the t-loop, created by the telomere DNA folding back on itself to form a lariat whose leading end is the telomeric 3' G strand overhang. This is envisaged as invading adjacent duplex telomeric repeats, thus creating a displacement loop (D-loop). Duplex DNA binding proteins are proposed to bind along the telomeric repeats of the t-loop, while a specialised DNA binding protein stabilises the D-loop lariat junction (Griffith et al., 1999).

Recently a protein capable of binding the single stranded G-rich extension found at the ends of all telomeres has been identified (Baumann and Cech, 2001).

13

This protein has been termed POT1 (Protection of Telomeres) and appears conserved in eukaryotes as diverse as yeast and humans. Deletion of the fission yeast pot1 gene produces immediate chromosome instability and rapid loss of telomeric DNA. The capping of telomeres thus appears to be crucial for avoiding chromosome instability and descent into cell death. In telomerase negative human somatic cells, telomeres may shorten with each cell division, finally reaching a critical level. However, cell death will not be triggered unless the telomere loses its capping proteins and becomes unstable. The state of the telomere (capped or uncapped) may also determine the degree of shortening which occurs with each cell division. Therefore telomere binding proteins may contribute directly to the internal mechanism which determines when the cell enters senescence.

Oxidative damage

Free radicals (ROS) are known to promote DNA damage and hence to contribute to the accumulation of damage which can promote cellular senescence. Oxidative metabolism is further known to promote production of ROS, and so promote senescence. Indeed, it has further been found that slowing metabolism of organisms, for example by restricting food

supply, prolongs life span to some extent. A number of observations provide an insight into how ROS may speed the entry of a cell into senescence.

Firstly, increased oxygen tension accelerates telomere erosion in replicating human fibroblasts *in vitro* (von Zglinicki and Schewe, 1995). Secondly, it has been observed that telomeres accumulated a significantly higher frequency of single-stranded overhangs, gaps and single-stranded breaks than the bulk of the genome when human fibroblasts were subjected to chronic oxidative stress (Peterson et al, 1998). Conversely, when human vascular endothelial cells were grown with an oxidation-resistant type of ascorbic acid, they showed a decrease in rate of telomere shortening by 52-62%, and an extension of cellular life span when compared to controls (Furumoto et al, 1998).

One possible explanation for this is that the G rich base repeat of telomeres is particularly susceptible to damage from ROS. This has been observed in lower eukaryotes where disruption of telomere structure occurs as a result of such damage. It is also possible that DNA damage to genomic DNA precipitated by ROS results in vital telomere binding proteins being recruited from the telomeres to the site of genomic DNA as part of the repair mechanism. This

-15-

would leave the telomeres exposed and uncapped, and thus more susceptible to instability.

Human in vivo evidence for an acceleration of telomere shortening in conditions of oxidative stress can be found
5 be examination of telomeres of patients with respiratory chain disorders. These conditions are associated with an increased production of ROS due to faulty mitochondrial respiration, and telomeres in these patients were on average 1.5 kb shorter than those of controls (Oexle and
10 Zwirner, 1997).

Molecular mechanisms underlying pathogenesis of CAN

The above observations are consistent with a hypothesis in which oxidative damage can lead to a senescent phenotype, indicating that such changes underlie
15 the development of CAN. Such damage is particularly relevant to transplantation and grafting, where the donor tissue may have been subject to ischaemic and hypoxic stress, with consequent changes in metabolism and production of ROS. Further relevant observations include:

- 20
- The presence of senescent features such as telomere erosion in cloned sheep (Shiels et al. 1999a, b) despite the animal appearing physiologically normal is in keeping with a model of senescence based on

oxidative damage.

- Age related changes have been observed in mitochondrial DNA sequence and conformation (Kopsidas et al, 2000) along with an altered DNA end binding capacity, significantly, mediated through

XRCC5 (Coffey and Campbell 1999).

- Mitochondrial xanthine oxidoreductase, a known cause of ischaemic reperfusion damage shows altered expression with age, resulting in elevated mitochondrial oxidative stress levels (Chung et al, 1999)..

- In vivo murine gene expression analysis and in vitro models of interstitial fibrosis indicate specific perturbation of the genes for telomere binding proteins, anti oxidant enzymes, and ribosomal components, in common with senescing cells.

- Mutations altering the function of telomere binding proteins can result in progeria (Myung et al, 2000).
- Specific age related changes in the expression of genes involved in the formation of the cytoskeleton and the extracellular matrix are also observed in kidneys undergoing CAN (Linskens et al, 1995).

At a molecular level, much of the damage of a

prolonged cold ischaemic time will result directly from the ROS produced when hypoxic cells are reoxygenated. An increase in chronic rejection of transplants has been observed to correlate to prolonged cold ischaemic times.

5 ROS can accelerate cellular senescence directly by the mechanisms described above. Therefore the degree of cellular senescence already present within the donor tissue will become important, representing the degree of reserve that the cells have. Those cells that are nearer the end of
10 their life span (for example, with shorter telomeres) will be more susceptible to damage from ROS and will enter senescence faster.

Without wishing to be bound by theory, the present inventors believe that oxidative stresses affecting
15 transplant rejection result in changes in the production of ROS, and consequently the expression of telomere binding proteins. In particular, expression changes in G22P1, XRCC5, hPOT1, and SIRT2 have been investigated. These
20 proteins are involved in the maintenance of telomere structure, DNA repair, and silencing of subtelomeric regions.

G22P1 and XRCC5 monomers form part of a complex that was originally identified as a major autoantigen in

patients with autoimmune diseases such as scleroderma. It is known to bind the ends of double-stranded DNA with high affinity and to be involved in repair of DNA by NHEJ. They are also known to be specifically co-located at the telomeres with SIRT2 and Rap1, and to play an important role in telomere maintenance in yeast. These products are known to interact with the Werner helicase protein, and are also responsible for the silencing of genes adjacent the telomere (Nugent et al 1998) and are found along with SIRT2 along this silenced region next to the telomere. In yeast, loss of the analogous heterodimer, Ku, from the telomere promotes loss of SIRT2, for example when Ku is recruited to repair a double stranded DNA break.

It is believed that the expression of telomere binding proteins is diagnostic for CAN and graft suitability. The present inventors suggest that DNA damage accrued pre-transplant results in substantial upregulation of G22P1 and XRCC5 expression, with concomitant telomere destabilisation as their respective gene products are recruited to DNA breaks elsewhere in the chromosome. Increase in hPOT1 expression occurs in an attempt to offset the destabilisation. Further, enhancement of expression of these proteins in the pre-transplant tissue would be

19

expected to reduce the risk of ROS damage to the cell, and hence lessen the risk of subsequent tissue rejection. Post-transplant intervention may also be possible to lessen the risk of subsequent damage and hence rejection.

5

Materials and Methods

RNA Isolation. Small frozen biopsy (10-50 mg) samples from human allografts were used to prepare RNA using Trizol (Sigma-Aldrich Co. Ltd, Poole, UK) following manufacturer's recommendations.

10

Samples were homogenised in Trizol using a PowerGen 125 Tissue Homogenizer (Fisher Scientific) using a test tube pestle. The homogenisation was performed until a homogeneous solution was obtained and very few visible tissue pieces were present. Samples were then incubated at room temperature for 5-10 minutes and the homogenate transferred to a centrifuge tube, and centrifuged at 12,000g (10,000 RPM on SS34) for 5-10 minutes at 4°C.

15

The upper layer was removed using a Pasteur pipette and 0.2 ml chloroform added per 1 ml Trizol reagent used initially. The tube was shaken vigorously for 15-30 seconds, then incubated at room temperature for 5 minutes. Samples were then spun at 12,000g for 15 minutes at 4°C. The upper aqueous phase, which contains the total RNA, was

20

20

removed and placed in a new centrifuge tube.

The RNA was precipitated by adding 0.5 ml isopropyl alcohol per 1 ml Trizol reagent used, and then incubated at room temperature for 10 minutes, followed by centrifugation

5 at 12,000g for 10 minutes at 4°C. The supernatant was then

~~decanted and the pellet washed once using 70% ethanol and~~

spun at 7,500g (7,500 RPM in SS34) for 5 minutes at 4°C. The

supernatant was again decanted and the pellet air dried

briefly. The RNA pellet was then resuspended in 200 µl DEPC

10 water, and quantified by spectrophotometry.

Slot blotting. The slot blot apparatus was assembled using Hybond N+ nylon membrane (Amersham, UK) as the solid support in accordance with the manufacturer's instructions, particular care being taken that no leakage occurred

15 between wells before loading the RNA samples. This was

tested using Orange G in sterile water as a marker dye. The

wells were rinsed twice with 10 x SSC and fluid sucked through the manifold under vacuum. The vacuum was then

turned off, and a further 50 ml of 10 x SSC added to each

20 well. Two volumes of RNA loading buffer was added to each

5 µg sample of RNA, which were then denatured at 70°C for 10

minutes, and chilled on ice. RNA samples were then loaded

into the wells already containing 10 x SSC and aspirated.

through under vacuum. The wells were then rinsed twice with
200 µl of 10 x SSC and this drawn through under vacuum. The
resulting slot blot was then rinsed in 2 x SSC and briefly
air dried, then the RNA UV cross linked in place and stored
5 at room temperature until required for hybridisation.

Hybridisation. This was performed at 65°C in 6 x SSC
overnight with the respective probes labelled with α -³²P.
Subsequent washing post-hybridisation was to a stringency
of 0.2 x SSC at 65°C. The blot was then subjected to
10 analysis using a Molecular Dynamics PhosphorImager.
Statistical evaluation of signals was conducted using
Microsoft Excel.

Generation of hybridisation probes. Probes were
developed by RT-PCR of human mesangial cell RNA, using
15 primers designed to amplify parts of the mRNA sequence of
the gene of interest. Primers for hPOT1 were selected
according to published data (Baumann and Cech, 2001).
Primer sets for SIRT2, G22P1 and XRCC5 were developed from
the respective GenBank sequence records using a specific
20 algorithm provided by Vector NTI suite (Informax). Primer
sequences are as follows:

1. XRCC5

Primer Sense: CTG AGG ACC GGC AAC ATG GT

Primer Antisense: CCA TCT TCC TTG CCA AGT GA

2. G22P1

5 Primer Sense: AAC ACG TCT CTT GCC AGG TC

Primer Antisense: CAC AAG TCT GGT GGT GGT GT

3. hPOT1

10

~~Primer Sense: GGA AGT GCA GCA GGG TTC AG~~

Primer Antisense: GGT TCT GCT TCC CTT TCA GT

15 4. SIRT2

Primer Sense: CCG CTA AGC TGG ATG AAA GA

Primer Antisense: GAC TGG GCA TCT ATG CTG GC

20

The level of gene expression was then measured by in situ hybridisation with the appropriate amplicons.

Experiment 1:

Consent was obtained to take a small biopsy (10-50 mg)

25 of renal tissue from the allografts at transplantation or

nephrectomy. RNA was extracted from this biopsy and 5 µg

transferred by means of a slot blot onto Hybond N+

membrane. Probes were developed by RT-PCR of human

mesangial cell RNA using primers as described above. The

30 level of gene expression was then measured by in situ

hybridisation with the appropriate α -³²P labelled probe.

Phospho-images of the hybridised blots were then developed

and analysed using ImageQuant technology.

A total of 4 samples from allografts with CAN, 4 from T_0 cadaveric donor biopsies, and 8 from T_0 living donor

biopsies was analysed on multiple Northern slot blots.

Comparative expression data from a representative blot is

5 illustrated in Figure 1. This indicates that there was an increase in the expression of G22P1 and XRCC5 in the CAN samples when compared to T_0 living and cadaveric samples.

Statistical analysis using a split-plot analysis of variance was undertaken to compare expression for each gene
10 in a) T_0 living and T_0 cadaveric samples; and b) T_0 living and CAN samples, with comparison being made between the three biopsy classes for the four genes tested. The raw data from the analysis is shown in Figure 2.

Overall kidney type differences were assessed by
15 comparison with the residual variation between samples and are significant at $p < 0.01$. Gene differences were assessed by comparison with the residual variation within samples and are significant at $p < 0.001$. Overall differences between kidney types with genes were assessed by comparison with a
20 combination of between and within sample variation, and are significant at $p < 0.05$. G22P1, XRCC5, and hPOT1 analysed within the respective groups showed significant differences in expression (at $p < 0.05$). Surprisingly, there was also a

significant increase in expression of SIRT2 in T₀ living samples when compared to T₀ cadaveric, despite no difference being observed between T₀ living and CAN samples ($p < 0.05$).

The increased expression of G22P1, XRCC5, and hPOT1 in
5 CAN biopsies indicates that cellular senescence is a
~~contributory factor in the pathogenesis of CAN.~~ The
reduction of SIRT2 expression in T₀ cadaveric samples when
compared to T₀ living samples is interesting, and may
reflect the mechanism by which oxidative damage promotes
10 cellular senescence and therefore CAN, as this was not
observed for the other three senescence associated genes.
The observed differences in expression data do not appear
to be correlated with the age of the donor organ, but
rather are a specific reflection of senescence associated
15 processes.

These results indicate that the G22P1, XRCC5, and
hPOT1 genes are early markers for the development of CAN,
and suggest a method for the screening of donor tissues
prior to transplantation. For example, if features of
20 cellular senescence, such as increased expression of G22P1
and XRCC5, are found in donor kidneys, it might be assumed
that these grafts are more likely to develop CAN than those
with low levels of expression of these genes. Routine

25

biopsies that indicated a sharp increase in the expression of G22P1 or XRCC5 and therefore the imminent development of CAN would contribute to the more precise management of the transplanted kidney, or to the suppression of the development of CAN. Further, the results of analysis of SIRT2 expression indicate that a decrease in SIRT2 expression in cadaveric samples may be prognostic for the future utility of cadaveric tissues. Enhancement of expression of all four genes may be expected to provide additional protein for cell and DNA maintenance and repair, and so prolong the effective lifespan of transplanted organs and reduce the risk of rejection.

REFERENCES

Baumann P, Cech TR (2001). Pot1, the putative telomere end-binding protein in fission yeast and humans. *Science*. 292:1171-5.

5

Chung HY, Song SH, Kim HJ, Ikeno Y, Yu BP.(1999).

Modulation of renal xanthine oxidoreductase in ageing: gene expression and reactive oxygen species generation. *J Nutr Health Ageing*. 3:19-23.

10

Coffey G and Campbell C (1999) An alternate form of Ku 80 is required for DNA end-binding activity in mammalian cells.

Nuc. Acids Res 28: 3793-3800.

15

Furumoto K, Inoue E, Nagao N, Hiyama E, Miwa N (1998). Age-dependent telomere shortening is slowed down by enrichment of intracellular vitamin C via suppression of oxidative stress. *Life Sci*. 63:935-48.

20

Griffith JD, Comeau L, Rosenfield S, Stansel RM, Bianchi A, Moss H, de Lange T (1999). Mammalian telomeres end in a large duplex loop. *Cell*. 14;97:503-14.

Halloran P, Melk A, Barth C (1999) Rethinking chronic allograft nephropathy: the concept of accelerated senescence. *Am Soc Nephrol*, 10:167-181

5

Kopsidas G, Kovalenko SA, Heffernan DR, Yarovaya N, Kramarova L, Stojanovski D, Borg J, Islam MM, Caragounis A, Linnane AW. (2000). Tissue mitochondrial DNA changes. A stochastic system. *Ann N Y Acad Sci*. 908:226-43.

10

Linskens MH, Feng J, Andrews WH, Enlow BE, Saati SM, Tonkin LA, Funk WD, Villeponteau B. (1995) Cataloguing altered gene expression in young and senescent cells using enhanced differential display. *Nucleic Acids Res*. 23:3244-51.

15

Myung K, Datta A, Chen C and Kolodner R. (2000) SGS1, the *Saccharomyces cerevisiae* homologue of BLM and WRN, suppresses genome instability and homeologous recombination. *Nature Genet*. 27: 113-116.

20

Nugent CI, Bosco G, Ross LO, Evans SK, Salinger AP, Moore JK, Haber JE, Lundblad V (1998). Telomere maintenance is dependent on activities required for end repair of double-

strand breaks. *Curr Biol.* 8:657-60.

Oexle K, Zwirner A (1997). Advanced telomere shortening in respiratory chain disorders. *Hum Mol Genet.* 6:905-8.

5

~~Paul LC (1999). Chronic allograft nephropathy: An update.~~

~~Kidney Int.~~ 56:783-93.

Petersen S, Saretzki G, von Zglinicki T (1998).

10 Preferential accumulation of single-stranded regions in telomeres of human fibroblasts. *Exp Cell Res.* 239(1):152-60.

Shiels PG (1999) Somatic cell nuclear transfer as a tool
15 for investigating ageing processes in Mammals *Gene Ther.*
Mol Biol 4: 11-22.

Shiels PG, Kind AJ, Campbell KH, Waddington D, Wilmut I,
Colman A, Schnieke AE (1999a) Analysis of telomere lengths
20 in cloned sheep. *Nature* 399: 316-317.

Shiels PG, Kind AJ, Campbell KH, Waddington D, Wilmut I,
Colman A, Schnieke AE (1999b) Analysis of telomere length

29

in Dolly, a sheep derived by nuclear transfer. *Cloning*

1(2):1-7.

von Zglinicki T, Schewe C (1995). Mitochondrial water loss
5 and ageing of cells. *Cell Biochem Funct.* 13:181-7.

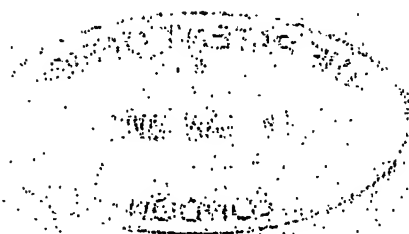


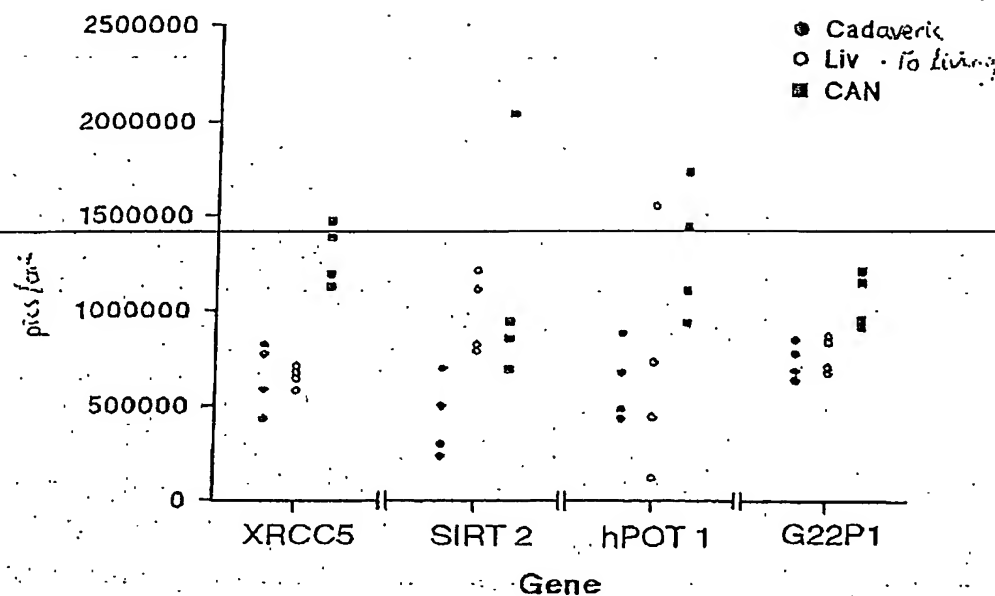
Figure 1

Figure 2

***** Analysis of variance *****

Variate: SQRTsig

Source of variation	d.f	s.s	m.s	v.r	F pr.
Sample stratum					
Kidney type	2	191398	95699	11.92	0.003
Residual	9	72228	8025	2.09	

Samples.*Units* stratum

Genes	3	3240289	1080096	281.32	<.001
KidType.Genes	6	59590	9932	2.59	0.041
Residual	27	103664	3839		
Total	47	3667169			

***** Tables of means *****

Variate: SQRTsig

Grand mean 505.3

Kidney type	Cadav	Live	Reject
	436.5	490.6	589.0

Genes	Gene1	Gene2	Gene3	Gene4
	924.3	350.1	506.9	240.1

KidType	Gene1	Gene2	Gene3	Gene4
Cadav	864.6	306.4	366.2	208.7
Live	882.5	308.0	563.6	208.2
Reject	026.0	435.8	590.8	303.5

*** Standard errors of differences of means ***

Table	KidType	Genes	KidType
	Genes		
rep.	16	12	4
s.e.d.	31.67	25.3	49.43
d.f.	9	27	31.64

The comparisons where there are significant differences (at $p < 0.05$),
are reject > live & live = cadav for gene 1

reject > live & live = cadav for gene 2

reject = live & live > cadav for gene 3 and reject = live & live = cadav for gene 4.

***** Tables of means *****

Variate: Signal

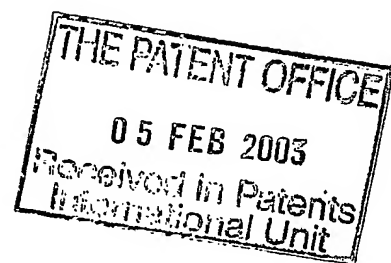
Grand mean 331772.

KidType	Cadav	Live	Reject
2	57252.	311656.	426409.

Genes	Gene1	Gene2	Gene3	Gene4
	861825.	126857.	275505.	62901.
KidType				
Cadav	749194.	95149.	140235.	44428
Live	780627.	94960.	320138.	50898.
Reject.	1055656.	190461.	366141.	93378

PCT/GB2003/000187

S. S.



**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.